Controllable Inactivation of a Toxin-based Tool to Target Actin *in vivo*

An Undergraduate Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* from The Ohio State University College of Arts and Sciences

By

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April 2015

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Table of Contents

Abbreviations

- G-actin: Monomeric (globular) actin
- F-actin: Filamentous actin
- BPM: Benzophenone-4-Maleimide
- Photo-L: Photo-leucine
- ACD: Actin crosslinking domain
- Cys: Cysteine
- Sup: Supernatant
- DMF: Dimethylformamide
- DTT: Dithiothreitol

ABSTRACT

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The actin crosslinking domain (ACD) is an effector domain of the larger MARTX toxins produced by the Gram-negative bacteria *Aeromonas hydrophila, Vibrio cholerae,* and *Vibrio vulnificus*. ACD, an actin specific toxin, catalyzes the covalent crosslinking of actin via formation of intermolecular isopeptide bonds between the K50 and E270 residues of actin monomers. The resulting actin oligomers vary in size and are non-polymerizable. It is a widely accepted theory that ACD toxicity stems from direct disruption of the actin cytoskeleton by depleting monomeric and filamentous actin pools, leading to the accumulation of non-polymerizable actin oligomers. Due to the high selectivity of ACD for actin, this toxin is an attractive candidate for developing a tool to study actin *in vivo*. However, a method to inactivate ACD inside the cell does not currently exist.

One potential solution is to use UV-induced crosslinking proteins containing photo-sensitive amino acids. I hypothesize that substituting leucine residues in ACD with their photo-sensitive, artificial analog photo-leucine (photo-L) will allow for the recombinant expression of functional ACD that can be inactivated upon UV light exposure. This will be achieved by expressing ACD in the presence of photo-L. Future steps are to utilize the anthrax toxin translocation system (LF_N domain and protective antigen) for delivery of the photo-controllable ACD to the cytoplasm of cultured cells where the ACD activity can be controlled by simple exposure of the cells to light.

Another potential solution is to modify ACD using a covalently bound photo-activatable crosslinker to produce ACD susceptible to UV-induced crosslinking. Benzophenone-4-maleimide (BPM) specifically and spontaneously attaches to thiol groups characteristic of cysteine residues and is a good candidate crosslinker for such an approach. Cys-mutantions must be introduced into ACD because the wild-type protein lacks sulfur-containing cysteine residues. To this end, ACD Cys-mutants were generated via multisite-directed mutagenesis in order to produce a library of His-tagged Cys-mutant LF_NACD_{Vc} which were expressed in *E. coli* prior to being labeled with BPM. A convenient reporter for the ACD toxin activity is covalent crosslinking of actin, which can be visualized using SDS-gel electrophoresis as an accumulation of higher molecular weight products of actin crosslinking in the presence of active ACD. Preliminary results suggest that the introduced Cys-mutations affect the activity of ACD even in the absence of BPM-labeling. However, recent results reveal that newer sites targeted for Cys-mutation in ACD did not abolish the toxin's activity. With this achieved, the method should become a tool to allow on-demand ACD inactivation to further understand its pathogenicity and to study actin functions *in vivo*.

Acknowledgements

As a member of the Kudryashov Lab at The Ohio State University Department of Chemistry and Biochemistry for one year, I have received extensive instruction and guidance from some great mentors. I would first like to thank Dr. Dmitri Kudryashov for welcoming me into the Kudryashov Lab, providing me with all the tools necessary to carry out my project, as well as mentoring me and encouraging me throughout my research. He is very driven and professional, and he molds the members of his lab into scientific problem solvers. Additionally, I would like to thank Dr. Elena Kudryashova, a research scientist in the lab, for her intellectual guidance, support, and mentorship throughout my research. I would also like to thank David Heisler, a graduate student in the Kudryashov Lab, who has been a great trainer of countless laboratory techniques and instructor of countless biochemical concepts during my research. Finally, I would like to thank all other members of the Kudryashov Lab for the valued intellectual and technical assistance they have provided me over the course of the past year.

1. Introduction

1.1 Actin in the cell

Actin is a highly conserved protein across many eukaryotic species. Found as a major protein component in various cell types, actin itself retains one important function: to polymerize into filaments. Actin can be found in the equilibrium between the two states described as monomeric (G-actin) and polymerized, filamentous (F-actin).[1] Actin works in conjunction with many other proteins such as actin-related proteins and those of the formin family to polymerize into filaments critical to the structure and function of the actin cytoskeleton of which reconstruction is paramount for cell motility, morphogenesis, and division.[2]

The dynamics of actin is to be in perpetual flux between both G-actin and F-actin states in order to provide semi-flexible filament polymerization and depolymerization in areas of the cell necessary for providing scaffolding for microvilli, lamellipodia and filopodia, tracks for intracellular transport, and providing the force necessary for motility on both the microscopic and macroscopic level.[1,2] The actin cytoskeleton of any cell is continually regulated and under scrutiny by countless other proteins, and when the actin cytoskeleton is disrupted, cell death is almost certain.[1,2]

1.2 ACD's role in bacterial pathogenesis

The actin crosslinking domain (ACD) (Figure 1) is an effector domain of the MARTX and VgrG1 toxins produced by the Gram-negative bacteria *Aeromonas hydrophila, Vibrio cholerae,* and *Vibrio vulnificus*. ACD is an important virulence factor of ACD-producing bacteria as it helps to evade host immune responses by disabling macrophages. ACD, an actin-specific toxin, catalyzes the covalent crosslinking of actin via formation of

intermolecular amide bonds between the side chains of K50 and E270 residues of actin monomers. The resulting actin oligomers vary in size and are non-polymerizable. According to the current paradigm, ACD toxicity stems from direct disruption of the actin cytoskeleton by depleting monomeric and filamentous actin pools, leading to the bulk accumulation of non-polymerizable actin oligomers, cell rounding, and eventually cell death.[3,4]

In contrast to the previously accepted view, the Kudryashov lab has found that only small amounts of oligomers may in fact be very toxic to the cell. However, currently no method for on-demand inactivation of ACD exists; therefore no method for *in vivo* testing of low concentrations of actin oligomers exists, and such a hypothesis remains unable to be directly tested. This thesis is focused on exploring a method for developing photo-deactivatable ACD in order to gain spatiotemporal control over ACD's activity. Two approaches are investigated in this work: 1) using photo-sensitive amino acid, photo-leucine; 2) employing photo-activatable crosslinker, benzophenone-4-maleimide.

1.3 Photo-leucine as a crosslinker

L-2-amino-4,4-azi-pentanoic acid, more commonly known as photo-leucine (photo-L) (Figure 6), is an artificial leucine analog that contains a diazirine ring in its side chain capable of activation upon exposure to UV radiation to form a highly reactive intermediate useful for crosslinking to the side chains of nearby amino acid residues. When eukaryotic cells are grown in media containing photo-L, this photo-sensitive, artificial amino acid analog has been demonstrated to incorporate into expressed proteins at sites normally occupied by leucine residues.[5] However, use of photo-L in a prokaryotic expression system has never been reported. If successful, this method will allow for the recombinant

expression of functional ACD that can be inactivated in the cells by crosslinking upon UV radiation exposure.

1.4 Benzophenone-4-maleimide as a crosslinker

Benzophenone-4-maleimide (BPM) is a photo-activatable crosslinker that can be attached through thioether linkage specifically to sulfhydryl groups such as those found in cysteine (Cys) residues via spontaneous thioether linkage under physiological conditions. When illuminated with UV radiation, BPM produces an highly reactive, free radical intermediate, which is susceptible to crosslinking with the side chains of nearby amino acid residues.[6] Careful selection of the crosslinking sites on the toxin should preserve the ACD activity upon BPM labeling but lead to protein inactivation upon crosslinking induced by the UV irradiation.

1.5 Thesis Overview

The ACD orthologs used in experiments described throughout this thesis are those produced by *Vibrio cholerae* and *Aeromonas hydrophila* (and therefore, are referred to as ACD_{Vc} and ACD_{Ah}, respectively).

In this work, I expressed LF_NACD_{Ah}, a construct of ACD fused C-terminally to the 300-residue N-terminal domain of lethal factor (LF_N), a *Bacillus anthracis* toxin. LF_N is a benign part of the toxin capable of binding to *B. anthracis* protective antigen (PA), which can be used for intracellular delivery of ACD.[7] Photo-L-containing toxin was expressed using the pCold DNA Cold Shock Expression system (Clontech)[8] in minimal M9 media supplemented with photo-L. The recombinant protein was exposed to UV radiation. Actin crosslinking capabilities were compared to LF_NACD_{Ah} unexposed to UV light by SDS-PAGE. Alternatively, LF_NACD_{Vc} was subjected to multisite-directed mutagenesis in order to

produce toxin that contained Cys residues necessary for BPM labeling. After labeling, mutant LF_NACD_{Vc} was subjected to UV light and actin crosslinking was compared with that in the absence of UV exposure, again by SDS-PAGE. By developing a form of ACD inactivatable upon UV irradiation, I expect to produce a tool that will allow for further study of ACD's toxicity.

In the following chapters, I will describe detailed methodology for the LF_NACD expression, mutagenesis, and actin crosslinking assays (used to assess ACD activity). I will then explain the implication of my results and discuss some of my project's challenges. Finally, I will propose future actions to take for continuing this project, and I will discuss the potential significance of this project and the importance of the study of ACD.

2. Results

2.1 ACD retains crosslinking activity following expression in M9 media supplemented with photo-L

As a first approach to achieve the main goal of producing photo-inactivatable ACD, LF_NACD_{Ah} (64 Leu residues) was expressed in *E. coli* BL21 (DE3) pLysS using M9 amino acid drop-off media supplemented with either leucine or photo-L (Figure 8). Actin crosslinking is a convenient indicator for ACD activity, even in bacterial cell lysates without protein purification. Small-scale expression allows for processing simultaneously up to 24 samples, allowint the rapid analysis of several conditions for protein expression at a time. After small-scale expression in the dark using the pCold DNA Cold Shock Expression system (Clontech), bacterial cell lysate samples were exposed to 365 nm UV radiation at 15 cm for 30 minutes. Those samples as well as those unexposed were added to actin for *in vitro* crosslinking assays for analysis by SDS-PAGE (Figure 9). Actin oligomers formed in the presence of ACD did not separate into monomers under the SDS-conditions, and this confirms the oligomers' covalent nature and suggests that the toxin remained active even after treatment with UV light.

2.2 *In vitro* actin crosslinking results are ambiguous as to whether photo-L was incorporated into protein expression

Actin oligomers were observed to form with toxin expressed in high amounts of photo-L. Actin crosslinking capability was not diminished in samples treated with UV light. Analysis of the cell lysates revealed that LF_NACD_{Ah} was successfully expressed in both cells cultured in media containing leucine as well as in media containing photo-L. The toxin was found in the lysate supernatants (Figures 8, 10). This suggested that the expressed protein was capable of proper folding to be soluble in cell cytosol, which would be useful for future

in vivo assays. UV-treated lysate supernatants were visible on the gel, suggesting that UV treatment did not induce protein degradation in expressed LF_NACD_{Ah}.

Analysis of the SDS-PAGE for respective actin crosslinking activity assays revealed that expressed LF_NACD_{Ah} retained activity, even when expressed in the presence of photo-L without natural leucine. Surprisingly, all UV-treated LF_NACD_{Ah} retained actin crosslinking capability. This result indicates that despite successful protein expression under strict conditions of luecine deficit, the cells may still have had managed to produce leucine from catabolic products of glucose present in the media, which would suggest that photo-L may not have been incorporated. Therefore, we employed leucine and isoleucine auxotrophic *E. coli* cells (ML2, a C43 (DE3)-based strain) for LF_NACD expression. These cells were considered Ile auxotrophs because they lacked the *ilvE* gene, which coded for a transaminase necessary for the biosynthetic pathway employed to synthesize Ile. Additionally, growth in media containing 0.4-1 mM Tyr, suppressed the *tyrB* gene, rendering them unable to synthesize leucine and therefore act as Leu auxotrophs. Use of the ML2 strain of *E. coli* in this study was done so in order to control leucine and photo-L incorporation during protein expression.[9]

SDS-PAGE analysis of the LF_NACD expressions from ML2 cells revealed that cells grown in the presence of photo-L were not able to express LF_NACD_{Ah} perhaps because existing tRNA does not recognize photo-L. Interestingly, cells grown without leucine or photo-L and only with isoleucine were able to express LF_NACD_{Ah}. This suggests that perhaps basal levels of protein degradation provided enough leucine for LF_NACD_{Ah} expression or that perhaps cells used were able to synthesize their own leucine from glucose catabolites in the media and that methods used were therefore not optimized for

the ML2 cell line. Continued inability to produce a positive result lead to the exploration of another approach, one that centered on the photo-activatable crosslinker, BPM.

2.3 ACD retains crosslinking activity following mutagenesis of select residues to cysteine

Since wild type ACD lacks any Cys residues, multisite-directed mutagenesis was performed on various sites to produce a library of His-tagged Cys-mutant LF_NACD_{Vc} In order to label with BPM. A "semi-high-throughput" screening method was employed in which multisite-directed mutagenesis yielded a plethora of mutant toxins carrying various combinations of the mutations introduced using the designed primers. Under this method, up to twenty-four mutant toxins at once were able to be labeled with BPM, purified, UV irradiated, and tested for actin crosslinking capabilities. Plasmids encoding toxin capable of actin crosslinking were sequenced, allowing for the development of a library of mutants with various actin crosslinking abilities.

Primers for the first round of LF_NACD_{Vc} mutagenesis were designed so to mutate residues in close proximity of the ACD active site. The mutant toxins were labeled with BPM prior to being subjected to metal ion affinity chromatography using cobalt columns and eluted with imidazole in order remove other proteins and excess BPM and dithiothreitol followed by irradiation with 302 nm UV radiation. SDS-PAGE analysis for all mutants explored from the first round of LF_NACD_{Vc} mutagenesis revealed no expression concerns (Figure 11). However, upon examination of SDS-PAGE for subsequent actin crosslinking activity assays of respective protein expressions, no mutant LF_NACD_{Vc} produced was able to retain activity after just UV exposure or just BPM labeling only to have its activity abolished upon BPM labeling coupled with subsequent UV treatment (Figure 11). This is significant because mutants capable of doing so would be ones that are

spatiotemporally controllable through photo-inactivation and would be of interest for *in vivo* studies mentioned in *1.5*. Mutants that presented no activity under any experimental conditions were likely to have sites essential to protein function mutated to Cys. Mutants that lost actin crosslinking capability following BPM labeling most likely did so because BPM is a large, bulky compound that interfered with protein activity once attached to these sites. Therefore, primers were redesigned to avoid such scenarios, and another round of multisite-directed mutagenesis was performed.

2.4 Additional bulk from BPM does not interfere with ACD crosslinking activity at select residues

Considering that BPM is a sterically bulky molecule (Figure 4), it could be expected that its presence when attached to Cys residues near the active site may interfere with the activity of the ACD toxin. Therefore, primers for the second round of LF_NACD_{Vc} mutagenesis were selected to introduce Cys residues using the crystal structure of VgrG1 ACD (PDB: 4DTH, an ACD_{Vc} homolog) based on the following criteria: 1) distant from the ATP-binding cleft, 2) along the ACD/actin interface, 3) do not participate in formation of alpha-helix or beta-pleated sheet, and 4) preferably tyrosine or tryptophan in order to mimic the bulky ring structure of BPM. Several mutants generated upon second round of mutagenesis retained actin crosslinking capability after BPM labeling as judged by the SDS-PAGE analysis (Figure 13). This reinforces that additional bulk from BPM modification does not interfere with ACD crosslinking activity at select residues.

2.5 BPM labeling of LF_NACD_{Vc} carrying Y357C and D448C mutations results in inactivation of the toxin upon exposure to UV light

In order to achieve the main goal, Cys-mutants of ACD must retain their activity under the conditions of UV exposure and BPM labeling (when performed exclusively from

each other), but mutants lose activity when UV treatment follows BPM labeling. Mutants capable of doing that could potentially be labeled with BPM and then delivered to a cell for photo-inactivatable *in vivo* actin crosslinking. All of the mutant toxins except one lost actin crosslinking capability either after Cys replacement, labeling with BPM, or exposure to UV light before BPM labeling. However, the one potentially promising candidate, mutant "6," retained its actin crosslinking activity after all of these conditions, but its activity was abolished upon UV irradiation of the BPM form (Figure 13). Mutant "6" plasmid was sequenced to reveal that it contained Y357C and D448C mutations in the ACD sequence (Figure 14). This result implies that mutant "6" is a photo-inactivatable form of ACD, and that the main goal of this study has been met.

3. Discussion

Actin filament assembly and disassembly as achieved through G-actin/F-actin treadmilling is crucial to many functions of the cell including life of the cell itself. Therefore, disruption of the actin cytoskeleton has a high potential to be detrimental. ACD-mediated crosslinking of G-actin occurs via the formation of an amide bond between the lysine-50 residue of one monomer and the glutamate-270 of another, resulting in covalently crosslinked actin oligomers, which do not readily disassemble back into G-actin. Furthermore, because the oligomers may not participate in conventional actin filament polymerization due to geometrical constraints, catalytic activity of ACD in the cell, if left uninterrupted, depletes both the pool of G-actin that is normally readily available for F-actin polymerization and the pool of F-actin (due to actin treadmilling) by sequestering eventually all actin into oligomeric form. Implications of this occurrence are observed in cell rounding and detachment from substrate. However, to directly test our hypothesis that extensive G-actin pool depletion is not necessary for cell toxicity, a form of ACD that is deactivatable on-demand must be produced to be able to form controllable small amounts of covalently crosslinked actin oligomers inside the cell.

In the development of methods outlined in this thesis, considerations needed to be made for an approach that would offer swift, on-demand, complete inactivation of ACD. Existing methods of protein inactivation in a cellular context did not satisfy these parameters. For example, degrons, peptide domains commonly found at the terminal ends of proteins which they are linked, mark the protein for ubiquitination.[10,11] Degrons were not implemented in this study because they rely on proteasomal degradation, which would be slower than desired because it would not simultaneously target every molecule of ACD

in the host cell.[10,11] Another approach using light, oxygen, voltage domains (LOV domains) linked to ACD for photo-inactivation via UV radiation would result in a protein susceptible to reactivation after some time without UV irratiation.[12–14] However, a permanently deactivated toxin was desired for this study. Additionally, since expression of the toxin in a host would be highly toxic and uncontrollable, this study involved the expression of LF_NACD in a prokaryotic organism with the intention of future testing the toxin in eukaryotic cells. Therefore, neither degrons nor LOV domains were implemented because in order to be transported across the cell membrane using anthrax delivery machinery, all components fused to LF_N must be thermolabile and flexible. Fusion of pliable ACD with LF_N has already been demonstrated to cross the membrane and retain activity.[3] Fusing ACD with folded domains such as degron or LOV would almost certainly affect the deliverability of the toxin across membranes and negate the benefit of LF_N fusion for future *in vivo* studies.

Another existing method of altering protein activity, photo-caging, is a process in which UV light exposure activates the desired protein by catalyzing its removal from a "caging group," which when linked, functions to keep the protein inactive.[15–18] However, this process is performed for permanent activation rather than inactivation of the desired protein, so it was therefore not implemented in this study.

With that said, two approaches have been explored in order to develop photo-inactivatable ACD. First , I explored expressing the toxin from media supplemented with photo-L. We hypothesize that ACD containing photo-L in place of endogenous Leu residues might be inactivated upon UV irradiation. In this mechanism, photo-L diazirine rings would be activated to release N_2 and form a reactive carbine intermediate species susceptible to

intramolecular crosslinking. The latter would alter the protein's structure, thus rendering it inactive. Use of photo-L for the expression of crosslinkable proteins is relatively popular albeit when the expression is being performed in eukaryotic organisms.[5] However, photo-L has never been shown to be incorporated into protein expressed from a prokaryotic organism. The alternative approach outlined in this thesis centered around using BPM, a photo-activatable crosslinker, to label the toxin to turn it into a controllable tool for actin crosslinking. Unlike degrons, both of these approaches aim to render efficient control of the toxin as inactivation is to be achieved through UV exposure.

The work performed in this thesis was conducted to support of another study that intends to discover whether low ACD-mediated actin oligomers may themselves be very toxic to the cell. The Y357C/D448C ACD mutant produced through this thesis's experiments appears to be a photo-inactivatable form of ACD and thus a promising candidate for use in testing the adjacent study's hypothesis. Future investigation of the mutant *in vivo* is to be performed.

4. Materials and Methods

This chapter describes the experimental protocols employed to execute the research approaches discussed throughout the previous chapters. The first subchapter will outline those used in the approach of producing LF_NACD_{Ah} constructs using photo-L. The second subchapter will outline those used to produce LF_NACD_{Vc} mutants subjectable to BPM labeling.

4.1 Photo-L Protocol (Figure 5)

4.1.1 Expression of LF_NACD_{Ah} in presence of photo-L

pCold (Clontech) DNA plasmid encoding LF_NACD_{Ah} was transformed into either BL21 (DE3) pLysS or ML2 (a C43 DE3-based Leu-auxotrophic strain) competent cells. A single colony from the transformation was used to grow a culture of cells for 16 hours at 37 °C, shaking at 250 rpm. The culture was used to inoculate into 25 mL of MMI rich medium (tryptone 1.25%, yeast extract 2.50%, NaCl 1.25 mM, glycerol 0.40%, Tris-HCl 50 mM at pH 8.2) and cells were grown at 37 °C, shaking at 250 rpm until their optical density at 600nm was between 1.2 and 1.8 as determined using a spectrophotometer. Cells were washed with M9 salts (0.11M KH_2PO_4, 0.266M $Na_2HPO_4x2H_2O$, 0.043M NaCl, pH 7.2) before being resuspended in M9-amino acid drop off media (5X M9 salts 1:5, 100X BME Vitamins 1:100 (Sigma Aldrich), $MgSO_4$ 2mM, $CaCl_2$ 0.1mM, 20% D-glucose 1:50, 10% NH_4Cl 1:100) and transferred to tubes wrapped in aluminum foil in 2 mL aliqots. Then, culture samples were supplemented with leucine and Photo-L according to (Figures 8, 10). Cells were incubated at 15 °C, 250 rpm for three hours before the induction of the recombinant protein expression with 1mM IPTG followed by 16 hours of incubation at 15 °C, 250 rpm (according to the Clontech manual for pCold DNA Cold Shock Protein Expression system).[8]

Cells were resuspended in lysis buffer (Tris-HCl 20mM at pH=8, NaCl 500mM, imidazole 10mM, $MgCl_2$ 2mM, PMSF 0.5mM, Leupeptin 1:500, trypsin inhibitor 1:500, benzamidine 4mM) and lysed by freeze thawing (three cycles) with subsequent sonication (15x 1 second pulses at 60% amplitude). The lysates were fractionated by centrifugation (20,000 *xg* for 20 min at 4 °C). Pellet (insoluble) and supernatant (soluble) fractions were analyzed via SDS-PAGE (Figures 8, 10). Samples of lysate supernatants were subjected to 365nm UV light at 15cm for 30 min on ice.

4.1.2 Actin Crosslinking Activity Testing

A master mix for the reaction of ACD-mediated actin crosslinking was assembled (PMSF 0.1mM, EGTA 0.3 mM, $MgCl_2$ 0.1mM, ATP 200 μM, HEPES 50mM at pH 7.5, actin 5 μM, latrunculin B 6 μM, cell lysate at the ratio of 1:25 to actin) and the reaction was initiated by addition of $MgCl_2$ (1:10 to achieve 2 mM final). Time point aliquots were taken at 0 min, 2 min, and 15 min by mixing 10 μL of reaction solution with 10 μL of 2X SDS sample buffer. All samples were boiled for 5 min and analyzed by SDS-PAGE (Figure 9).

4.2 BPM Protocol (Figure 7)

4.2.1 Multisite-directed mutagenesis of LF_NACD_{Vc}

Primers were developed using http://www.bioinformatics.org/primerx/ that were at least 25 bases long, had a GC-content of at least 40%, and had melting points between 78 and 80 °C (Figures 2, 3). Primers were used simultaneously in the Lightning Quik-Change protocol for multi site-directed mutagenesis (Agilent Technologies) to produce mutant LF_NACD_{Vc} plasmids.[19]

4.2.2 Expression of LF_NACD_{Vc} mutants

Mutant LF_NACD_{Vc} plasmids were transformed into BL21 (DE3) pLysS cells. Replica plates for future plasmid sequencing were created for the resulting colonies before using the colonies for protein expression. Resulting colonies were used to inoculate into 5 mL of MMI rich medium and cells were grown at 37 °C, shaking at 250 rpm until their optical density at 600 nm was between 1.2 and 1.8 as determined using a spectrophotometer. The induction of the recombinant protein expression was initiated with 1mM IPTG followed by 16 hours of incubation at 15 °C, 250 rpm (according to the Clontech manual for pCold DNA Cold Shock Protein Expression system). Cells were resuspended in lysis buffer (Tris-HCl 20 mM, pH 8, NaCl 500 mM, imidazole 10 mM, $MgCl_2$ 2 mM, PMSF 0.5 mM, Leupeptin 1:500, trypsin inhibitor 1:500, benzamidine 4 mM) and lysed by freeze thawing (three cycles) with subsequent sonication (15x 1 second pulses at 60% amplitude).

4.2.3 BPM labeling

Lysate samples were labeled with BPM (0.4 mM final) in dimethylformamide, (DMF) and left to interact on ice for one hour before being treated with dithiothreitol (DTT, 4 mM final) to stop the labeling reaction.

4.2.4 Protein purification

After BPM labeling, cell lysates were subjected to metal ion affinity chromatography by loading onto Co^{2+} columns. Columns were washed with lysis buffer until washes yielded no protein as judged using Bradford Reagent (Bio-Rad). Isolated protein was then eluted using lysis buffer containing 250 mM imidazole.

4.2.5 Actin crosslinking activity testing

Purified protein samples from section 4.2.4. were subjected to 302 nm UV light for 15 min on ice. A master mix for the reaction of ACD-mediated actin crosslinking was

assembled (PMSF 0.1 mM, EGTA 0.3 mM, $MgCl_2$ 0.1 mM, ATP 200 µM, HEPES 50 mM, pH7.5, actin 5 µM, latrunculin B 6 µM, cell lysate 1:25) and the reaction was initiated by the addition of $MgCl_2$ (1:10 to achieve 2 mM final). Aliquots 10 µL were withdrawn at 15 min and mixed with 10 µL of 2X SDS sample buffer. Results were analyzed by SDS-PAGE (Figures 11, 13).

8. Figures

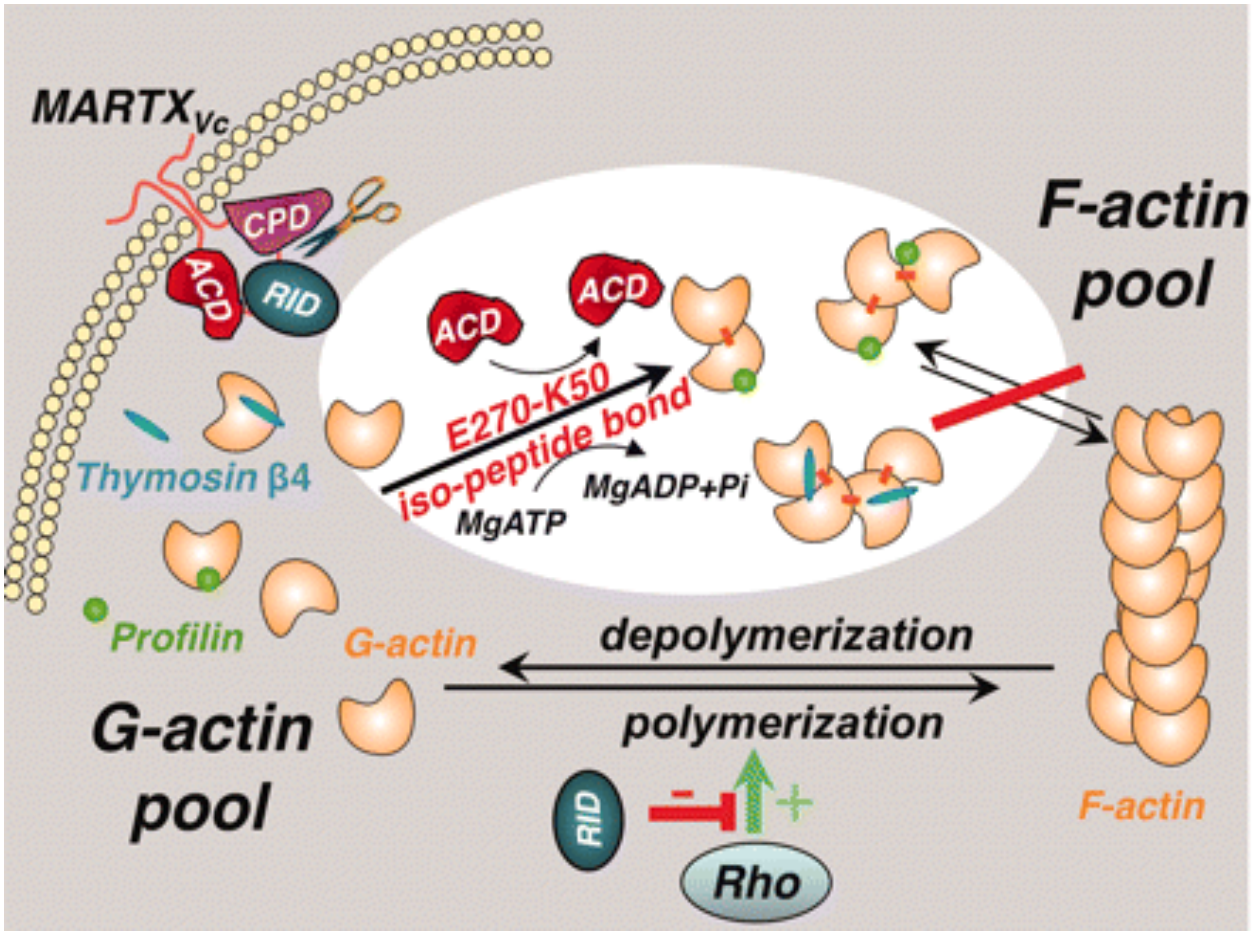


Figure 1: The actin crosslinking domain (ACD) covalently crosslinks G-actin via catalysis of isopeptide bond formation between E270 of one actin monomer and K50 of another, resulting in non-polymerizable actin oligomers. This depletes both the pool of G- and F-actin in the cell, disrupting functional and structural integrity of the actin cytoskeleton and negating motile abilities. Adapted from *Kudryashov et al., PNAS, 2008*. Copyright (2008) National Academy of Sciences.[4]

Mutations
S312C
T349C
N550C
I621C

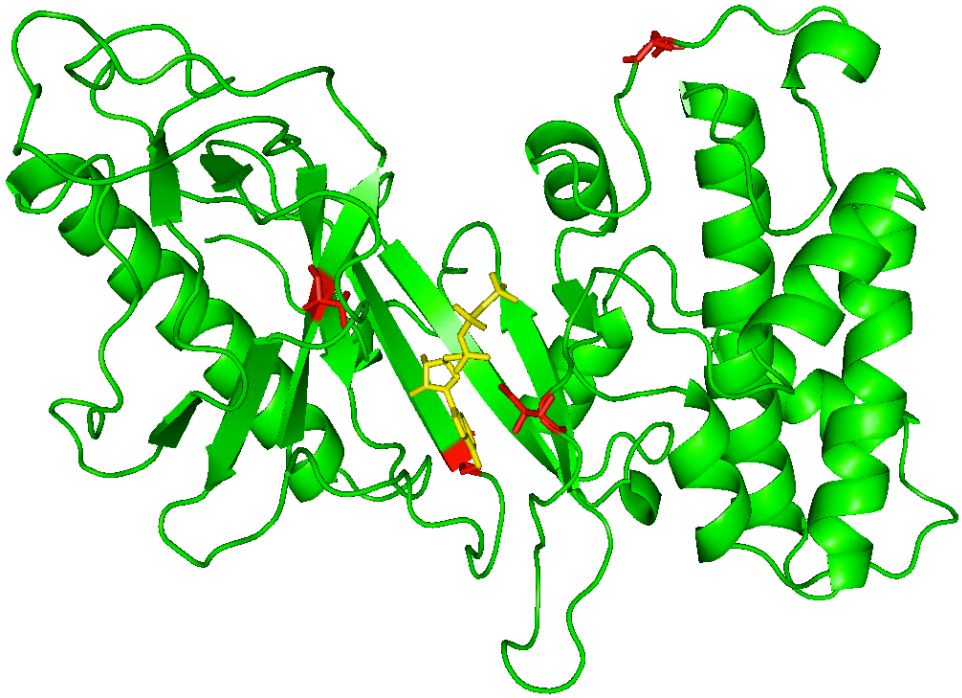


Figure 2: The crystal structure of VgrG1 ACD generated using pyMol. The yellow molecule is ATP. ACD is green, and residues targeted for round one mutagenesis are highlighted in red. The group of mutations chosen in this round were meant to include both sites near and far in reference to the ATP-binding cleft of ACD.

Mutations
Y357C
D448C
T457C
Y536C
N550C

Figure 3: The crystal structure of VgrG1 ACD generated using pyMol. The yellow molecule is ATP. ACD is green, and residues targeted for round two mutagenesis are highlighted in red. The group of mutations chosen in this round were done so by the criteria outlined in *2.4*.

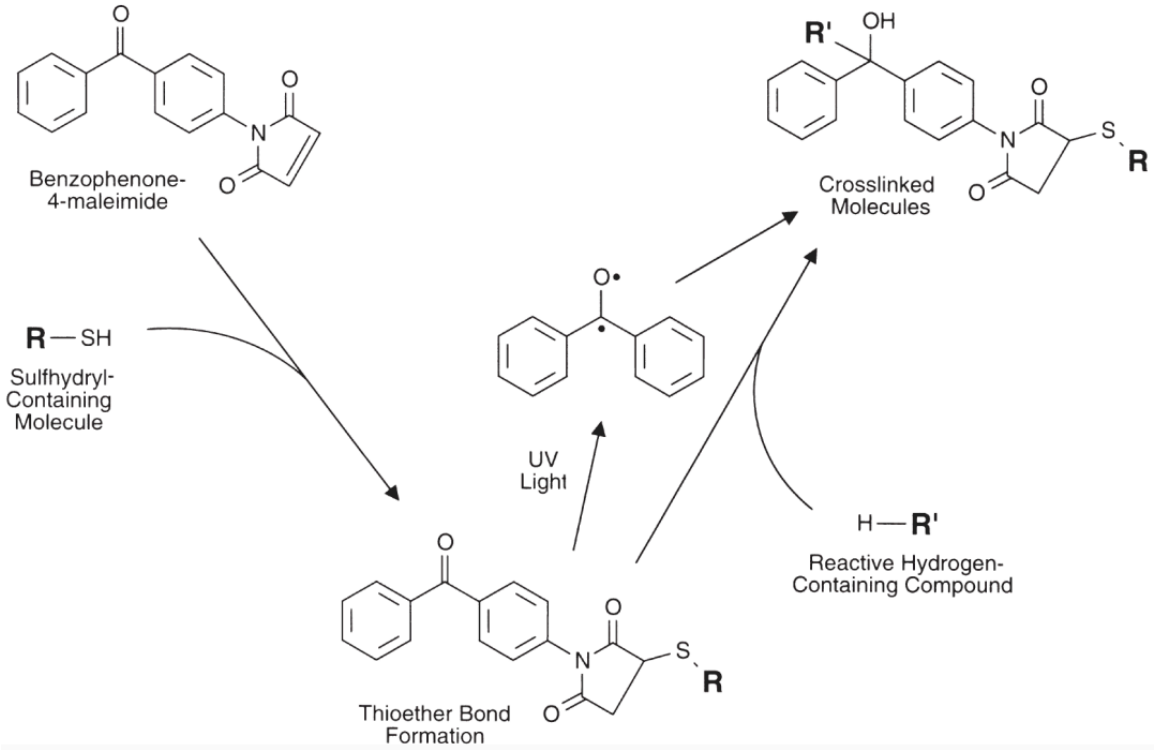


Figure 4: Benzophenone-4-maleimide's (BPM) crosslinking mechanism: BPM spontaneously links to sulfhydryl (thiol) groups to form stable thioethers. UV light catalyzes formation of a highly reactive radical carbene at the ketone alpha carbon susceptible for non-specific linking elsewhere within the protein. Cys residues were mutated into LF_NACD_{Vc} to provide thiol groups for BPM attachment in this study. Adapted from *Hermanson, 2013*. Copyright (2013) Elsevier.[6]

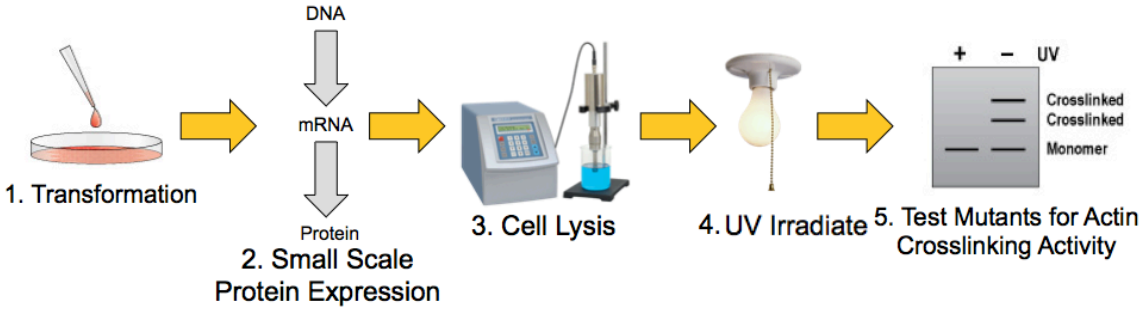


Figure 5: Scheme of the protocol for the photo-inactivation of ACD using LF_NACD expressed in media supplemented with photo-L

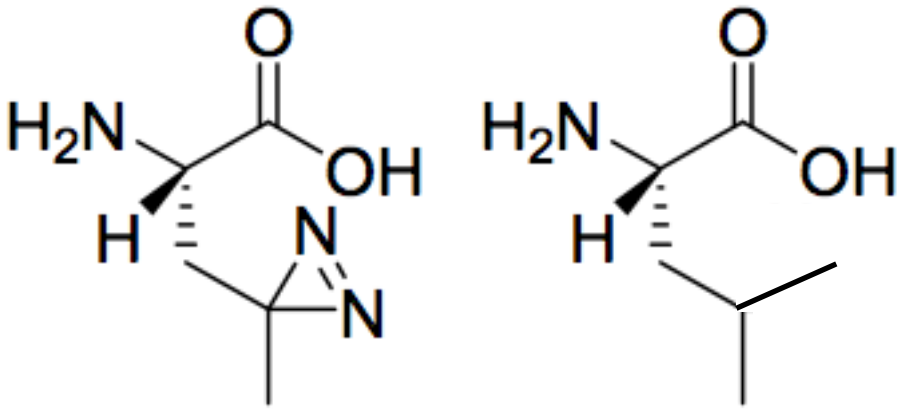


Figure 6: Comparison of photo-L (structure on left) and leucine (structure on right).

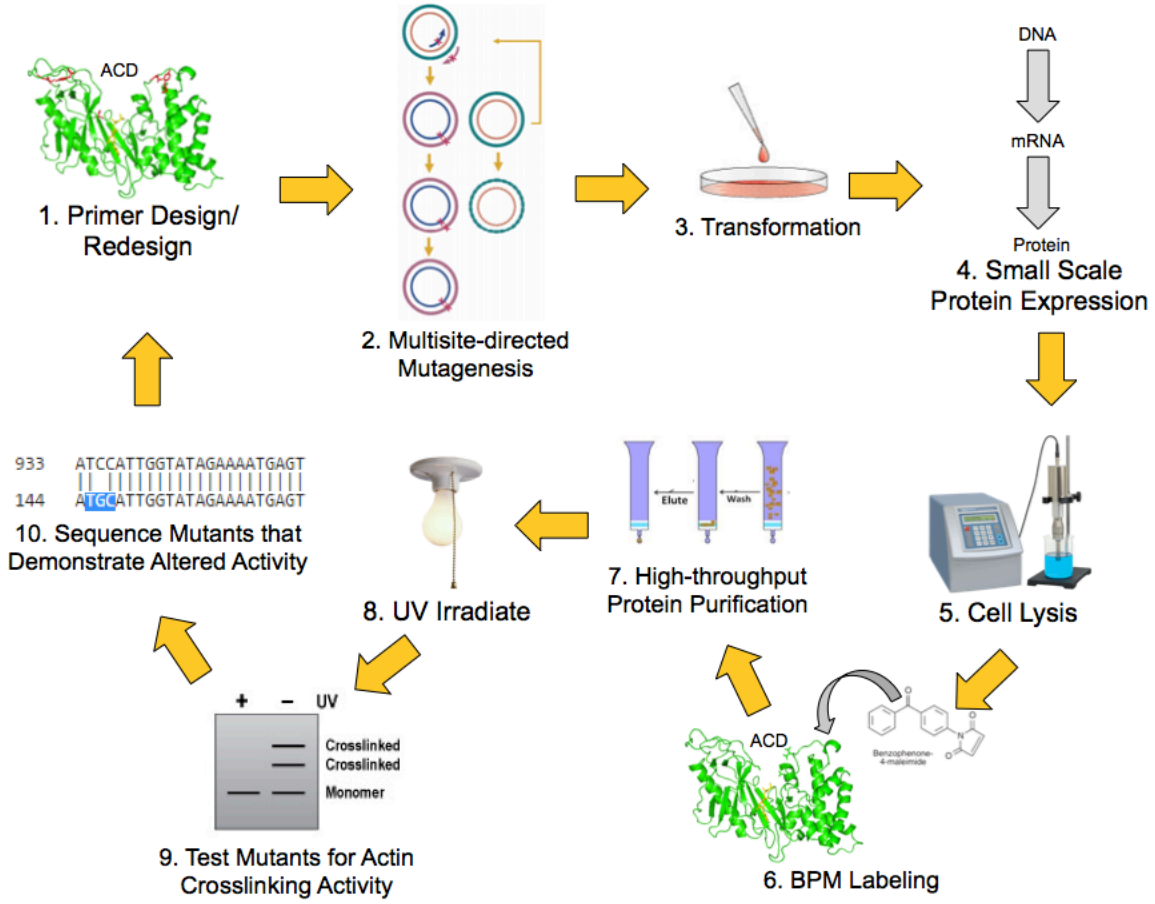
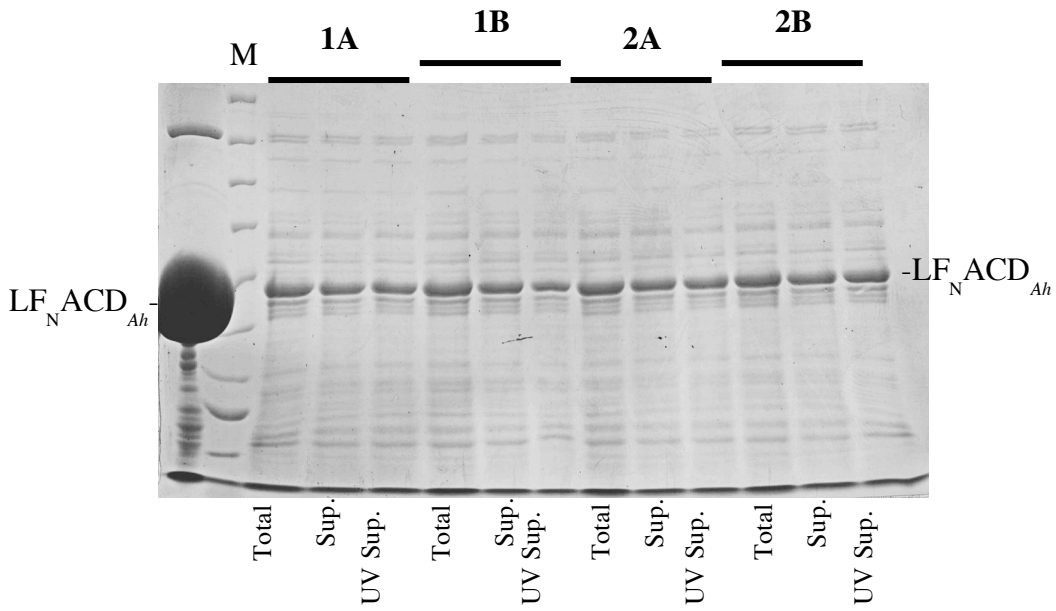


Figure 7: Scheme of the protocol for the photo-inactivation of ACD using LF_NACD_{Vc} mutants labeled with BPM



	1A	1B	2A	2B
Leucine	+	+	-	-
Photo-L	-	-	+	+

Figure 8: SDS-PAGE results for LF$_N$ACD$_{Ah}$ expression in the presence of photo-L. Expressed protein can be located in both soluble (supernatant) and insoluble (pellet) portions of the cell lysate. UV irradiation does not appear to degrade the protein. Media concentrations of leucine and photo-L for each condition are designated in the accompanying table.

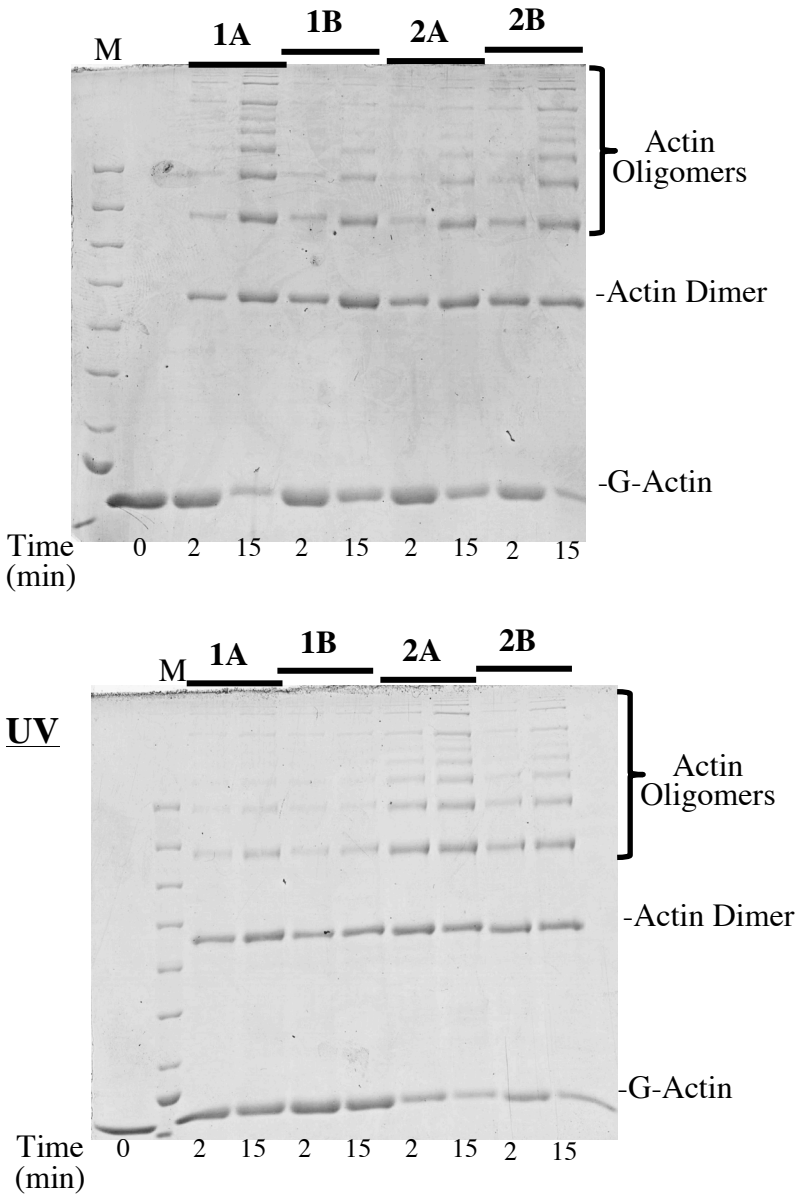
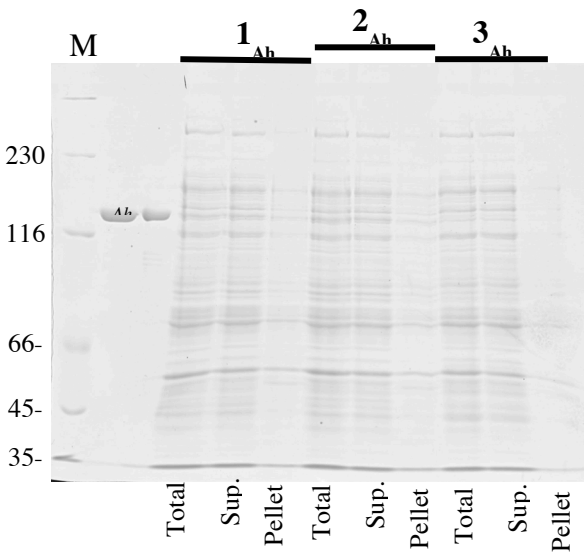


Figure 9: SDS-PAGE results for expressed LF_NACD_{Ah}; results for protein samples exposed to UV irradiation are depicted in the lower gel. Presence of oligomers in the lower gel suggests UV light does not appear to demolish ACD's actin crosslinking capability.



	1_{Ah}	2_{Ah}	3_{Ah}
Leucine	-	+	-
Photo-L	-	-	+

Figure 10: SDS-PAGE results for LF_NACD_{Ah} expression in the presence of photo-L. Toxin appears not to have expressed when in the presence of photo-L, and it cannot be found in the insoluble portion of cell lysate. Media concentrations of leucine and photo-L for each condition are designated in the accompanying table.

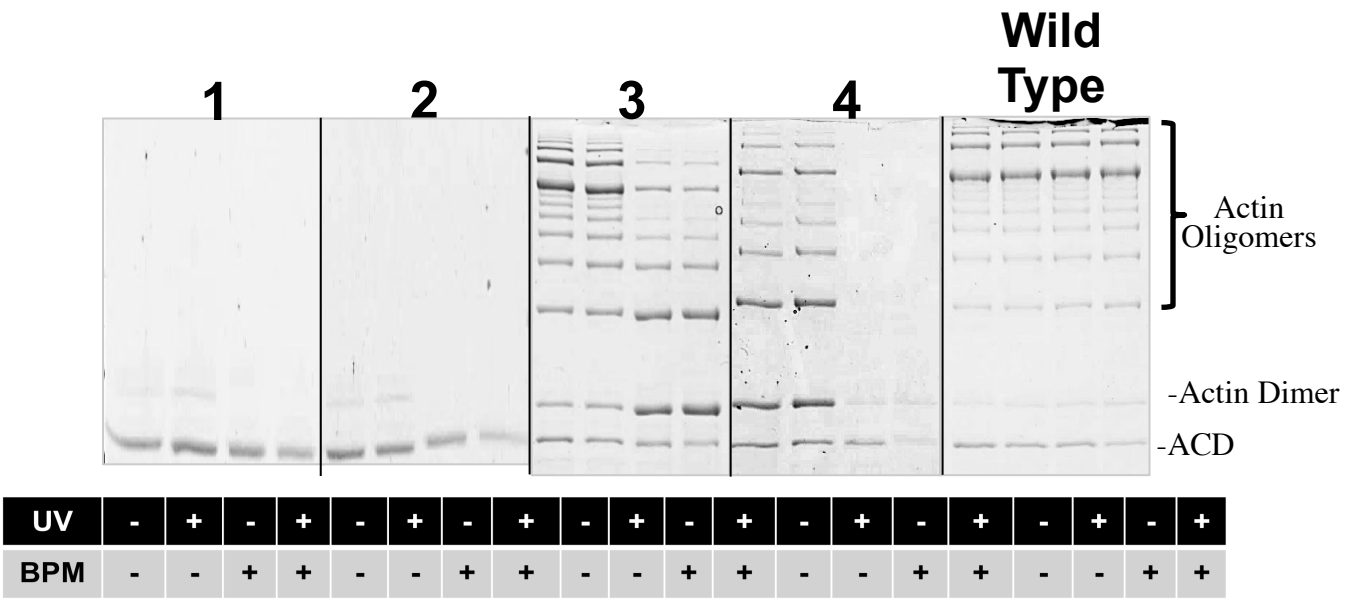


Figure 11: SDS-PAGE results for first-round LF_NACD_{Vc} mutants' actin crosslinking activity assays. None of the mutants 1, 2, 3, or 4 displayed retention of activity post-BPM labeling and the complete loss of activity following subsequent UV treatment.

Mutants			
Mutations	**1**	**2**	**3**
T312C	**YES**	**YES**	**NO**
T349C	**YES**	**YES**	**NO**
N550C	**YES**	**YES**	**YES**
I621C	**YES**	/	/

YES = mutation present
NO = mutation not present
/= Uncertainty due to sequencing errors

Figure 12: Table outlining Cys mutations identified following first-round of LF_NACD_{Vc} mutagenesis

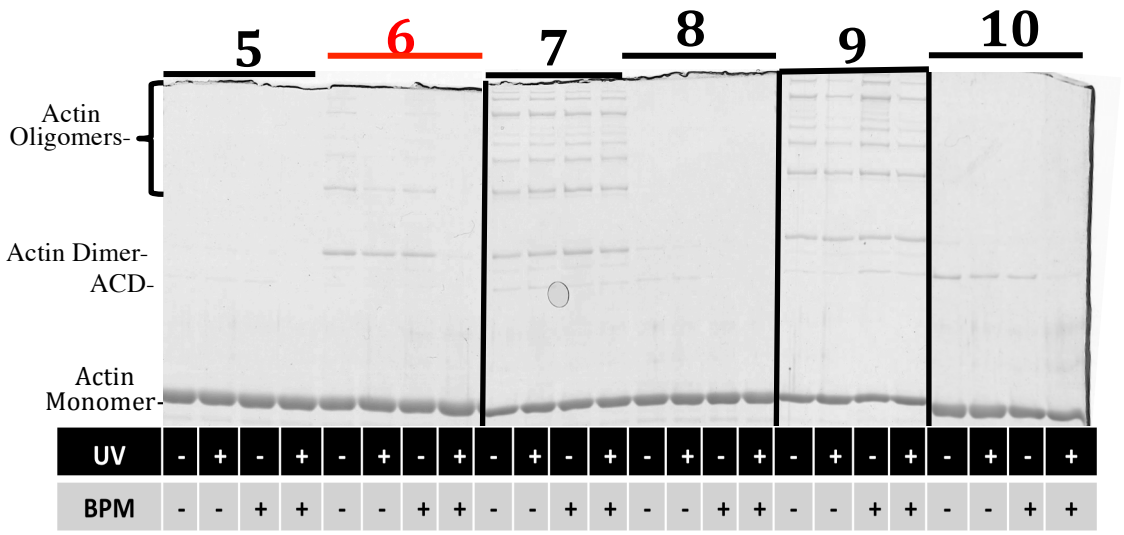


Figure 13: SDS-PAGE results for second-round LF_NACD_{Vc} mutants' actin crosslinking activity assays. A positive candidate, mutant "6", displays retention of activity post-BPM labeling and the complete loss of activity following subsequent UV treatment. The mutant "6" plasmid was sequenced to reveal Y357C and D448C mutations in the ACD sequence.

Mutants						
Mutations	**5**	**6**	**7**	**8**	**9**	**10**
Y357C	YES	YES	NO	YES	NO	YES
D448C	YES	YES	NO	YES	NO	YES
T457C	NO	NO	NO	NO	NO	NO
Y536C	YES	NO	NO	YES	NO	NO
N550C	NO	NO	NO	NO	NO	YES

YES = Mutation present
NO = Mutation not present
/= Uncertainty due to sequencing errors

Figure 14: Table outlining Cys mutations identified following second-round of LF_NACD_{Vc} mutagenesis

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